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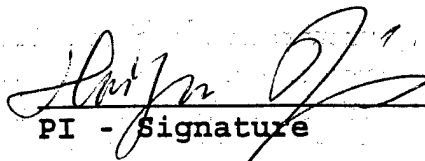
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## INTRODUCTION

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Nucleoside antimetabolites comprise one of the most effective classes of drugs used in the treatment of viral diseases and specific types of malignancy. The biological activity of most nucleoside antimetabolites is due to their ability to inhibit the DNA synthetic process, which is an essential function both for cell division and proliferation. 1- $\beta$ -D-arabinofuranosylcytosine (araC) has been used effectively in the clinic to treat hematologic cancers [5]. 2', 2'-difluoroxydeoxycytidine (gemcitabine, dFdC) is a novel deoxycytidine analog with structural and metabolic similarities to araC. Both of these compounds are prodrugs which are transported into the cell, where they are activated following phosphorylation by deoxycytidine kinase. AraC and gemcitabine differ in structure from the parent nucleoside, deoxycytidine, by specific modifications to the 2' carbon of the furanose ring. Clinical trials have shown that gemcitabine is effective in most solid tumors and more potent and less toxic than araC [12,13,10,18,20]. Intact cell studies have indicated that inhibition of DNA synthesis is the predominant effect of dFdC and araC [15,25,26,28]. Like araC, the major targets for dFdCTP are the DNA polymerases. It has been shown that incorporation of araCTP and dFdCTP into DNA is most likely the primary mechanism by which these drugs exert their cytotoxic effect [26]. Using *in vitro* DNA primer extension assays employing purified DNA polymerases, dFdCTP and araCTP have shown qualitative and quantitative differences in their molecular actions on DNA synthesis [15]. Studies comparing dFdC to araC showed that dFdC was transported more rapidly and is a better substrate for deoxycytidine kinase than araC [10]. Furthermore, gemcitabine inhibits ribonucleotide reductase and thus causes depletion of intracellular nucleotide pools [11]. AraC is not known to inhibit the activity of ribonucleotide reductase [11]. Finally, dFdC also showed a slower elimination rate than araC [10]. All of these characteristics of dFdC result in the development of higher intracellular concentrations of active metabolite (i.e., dFdCTP). Although inhibition of DNA synthesis has been strongly correlated with intracellular dFdCTP concentration [11], little work has been done to directly compare the effects of dFdCTP and araCTP on reducing the level of DNA replication within the cell.

We have previously reported that a highly purified multiprotein form of DNA polymerase (the DNA synthesome) can be isolated from a variety of mammalian cell types and tissues [2,3a,14,17,21,33]. We have shown that the DNA synthesome is fully competent to support origin-specific large T-antigen-dependent *in vitro* SV40 DNA replication [2,3a,21,33,]. Biochemical characterization of the DNA synthesome has identified several protein components of the complex that were found to be essential for DNA replication [14,23]. These proteins include the DNA polymerases  $\alpha$ ,  $\delta$ , and  $\epsilon$ , DNA primase, topoisomerases I and II, proliferating cell nuclear antigen (PCNA), replication factor C (RFC), replication factor A (RPA), DNA helicase, and DNA ligase I [2,21,33]. Most importantly, in the presence of viral large T antigen and the SV40 replication origin sequence, the synthesome is fully competent to carry out all phases of the DNA replication process required to replicate an SV40 origin containing plasmid *in vitro*. We have successfully examined the action of araC and

camptothecin using this model system [1,3b,9,31,32] and have now extended the results of these studies by exploring how the inhibitory effects of dFdC and araC compare with one another. Our studies compared the inhibitory effects of dFdC and araC on intact human breast cancer cell DNA synthesis and in DNA synthesis mediated by our *in vitro* DNA replication assay system. Our results demonstrated that dFdC was more potent in inhibition of *in vitro* DNA synthetic activity mediated by the DNA synthesome and in intact MCF7 cells than araC.

## SUMMARY OF RESEARCH

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### **A: Effect of dFdC and araC on intact MCF7 cell DNA synthesis:**

As proposed in task 1 of my statement of work, the first goal was to generate dose-response curves to determine the correlation between concentration of araC and dFdC with extent of inhibition of MCF DNA synthesome. In order to verify that araC and dFdC affect the ability of intact MCF7 cells to carry out DNA synthesis as previously reported [1,10,15]. Exponentially growing MCF7 cells were incubated in the absence (control assigned a value of 100%) or presence of increasing concentrations of araC and dFdC. The cells were exposed for 24 hours to drug concentrations ranging from 1 to 1000  $\mu$ M, and the drugs were then removed by washing the cells with PBS. Fresh media was added to the cell culture and the cells were incubated with [ $^3$ H]-thymidine for 4 hours. The labeled cells were lysed, and the level of DNA synthesis was measured by quantifying the amount of [ $^3$ H]-thymidine retained in acid-insoluble material. As shown in Figure 1, intact MCF7 cell DNA synthesis was inhibited by both drugs in a concentration-dependent manner. About 10  $\mu$ M dFdC and 80  $\mu$ M araC were required to reduce MCF7 cell DNA synthesis to 50% of the control activity measured in the absence of either drug. The IC<sub>50</sub> value for the inhibition of intact cell DNA synthesis by araC was comparable to that observed by us using the estrogen-receptor-negative breast cancer cell line, MDA MB-468 [1]. We also observed that the araC concentration required to inhibit 50% of intact HeLa cell DNA synthesis was also about 80  $\mu$ M (data not shown). This value was consistent with our previously reported IC<sub>50</sub> value for araC inhibition of intact cell DNA synthesis [22].

### **B: Effect of dFdC and araC on intact MCF7 cell clonogenicity:**

We next performed cell survival assays to compare the cytotoxic effects of dFdC and araC on the ability of MCF7 to form colonies. As shown in Figure 2, 50% of the cells lost their clonogenic capacity at concentrations of dFdC above 8  $\mu$ M following a 4-hour incubation of the cells with the drug. The concentration of dFdC needed to inhibit the clonogenic survival of 50% of MCF7 cells was approximately 12-fold less than that of araC. Thus, our results from the intact DNA synthesis and clonogenicity demonstrated that dFdC was significantly more cytotoxic to intact MCF7 cells than araC.

### **C: Isolation of DNA synthesome from human breast cancer MCF7 cells:**

In order to examine the effect of the drugs on the synthesome associated DNA synthetic activity, the DNA synthesome was first isolated from human breast cancer MCF cells using our published protocol essentially as described by Malkas et al [19,21] and as outlined in Figure 3. We designated the human breast cancer MCF7 cell DNA synthesome as M7DS. In my original proposal, the DNA synthesome from MCF-10A, which is a non-malignant breast cancer cell line, was also proposed to be used as a control for comparing inhibition effect of the two drugs. However, we did not include MCF-10A cells in our studies. One reason is that the panel questioned the relevance of these experiments to the overall project was uncertain. Another reason is that MCF-10A

cells is difficult to grow and turns to be malignant after several passage during cell culture process. Since we have synthesesomes from HeLa and MDA MB-468 cells as control, we therefore gave up using the synthesesome from MCF-10A cells.

**D: Comparison of the inhibitory effects of araCTP and dFdCTP on *in vitro* SV40 DNA replication using M7DS:**

We have previously shown that the DNA synthesesome isolated from the HeLa cell and human breast cancer cell MDA MB-468 is capable of supporting the origin-specific T-antigen-dependent SV40 DNA replication reaction *in vitro* [1,3b,31]. These studies demonstrated the utility of the purified DNA synthesesome as a relevant *in vitro* model that is useful for studying the mechanism of action of anticancer drugs such as: araC, camptothecin, and VP16. In order to directly compare the anti-DNA synthetic activity of araCTP and dFdCTP, we performed *in vitro* SV40 DNA replication assays in the absence and presence of several concentrations of each of these two drugs (1 $\mu$ M, 10 $\mu$ M, 100 $\mu$ M and 1mM). Both drugs inhibited SV40 DNA replication in a concentration-dependent manner as measured by quantifying the amount of  $^{32}$ P-dGTP incorporated into DNA (Figure 4). Fifty-percent inhibition of the *in vitro* DNA replication assay was achieved in presence of 10 $\mu$ M dCTP using approximately 8 $\mu$ M dFdCTP and 80 $\mu$ M araCTP. The results of this assay indicated that dFdCTP was able to more effectively compete with dCTP to inhibit DNA-synthesesome-mediated *in vitro* DNA replication than araCTP. In consistent with the results from the intact cell, dFdCTP showed more potent in inhibiting *in vitro* SV40 DNA synthesis than araCTP. Our data also showed a close correlation between the IC<sub>50</sub> values of both drugs for inhibiting intact cell DNA synthesis and the DNA synthesesome mediated *in vitro* SV40 replication assay.

**E: *In vitro* replication products produced by M7DS in presence of araCTP or dFdCTP.**

We further analyzed the replication products of the *in vitro* replication reaction using a 1% neutral and a 1% alkaline agarose gel (Figures 5 and 6, respectively). Our results indicated that the MCF7 cell DNA synthesesome was capable of producing full-length daughter DNA as shown by the presence of form I (superhelical) DNA and form II (nicked open circular) DNA, as well as higher-order replication intermediates (Figure 5 lane 2). This reaction was also T-antigen dependent (Figure 5 lane 1). In the presence of low concentrations (1  $\mu$ M) of both drugs, full-length daughter DNA molecules (form I and form II) were observed (Figure 5, lane 3 and lane 7). At higher concentrations, the production of form II DNA molecules as well as replication intermediates was inhibited in a concentration-dependent manner (note both neutral and alkaline gels (Figure 5, 6 lanes 2-10)). However, form I DNA molecules disappeared at drug concentrations higher than 1 $\mu$ M, indicating that dFdCTP and araCTP may impair the process to form supercoiled DNA. In the presence of both drugs, the production of short Okazaki fragments was inhibited in a concentration-dependent manner (Figure 6, lanes 3-10) and were completely inhibited at higher dFdCTP concentrations (Figure 6, lanes 5-6), suggesting that dFdCTP had a greater inhibitory effect on the initiation stage of DNA synthesis. These results were in accordance with our previously reported results using

the DNA synthesize isolated from HeLa cells and MDA MB-468 cells [1,22]. Furthermore, they correlate with other reports from this laboratory employing our DNA-synthesize-mediated *in vitro* DNA replication assay system as well as intact cells [15,27,28].

## CONCLUSION AND DISCUSSION

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Gemcitabine is a novel deoxycytidine analog with both structural and metabolic similarities to araC. AraC is one of the most effective drugs available today for the treatment of acute leukemia and other hematopoietic malignancies. Gemcitabine is also effective against leukemia, but it has proven to be effective against a variety of solid tumors. Like araCTP, dFdCTP inhibits DNA synthesis mainly through the inhibition of the activity of the DNA polymerases. A considerable body of work has accumulated in which the effects of these two drugs on intact cell DNA synthesis in a variety of cancer cell lines. Using a cell survival assay, Heinemann et al. [10,11] discovered, that Chinese hamster ovary cells are significantly more sensitive to dFdC than araC after both a 4- and an 18-hour incubation. Our study using the human breast cancer cell line MCF7 in a clonogenic assay also showed that dFdC is approximately 15-fold more cytotoxic than araC following a 24-hour incubation with the drug. Gemcitabine inhibits  $^3\text{H}$ -thymidine uptake approximately 10-fold more than araC in intact MCF7 cells. Accumulation of dFdCTP was observed to be cell line dependent, with the cell lines that are more sensitive to the drug accumulating higher amounts of dFdCTP in cultures [15,24]. This may explain the difference between the IC<sub>50</sub> values obtained in our experiments, and these obtained by other investigators who performed similar studies with CHO cells [10], human T-lymphoblastoid CCRF-CEM cells [15] and HL60 cells [28].

DNA synthesis is the most prominent activity inhibited by dFdC in cultured cell [10]. Huang et al. (1991) directly investigated the molecular mechanism of action of dFdC and araC *in vitro* on DNA synthesis using purified DNA polymerase  $\alpha$  and  $\epsilon$  [15]. However, the use of purified DNA polymerases may not adequately reflect the DNA synthetic process as it occurs within the intact cell. In the intact cell DNA synthesis involves the coordinated activity of DNA polymerase  $\alpha$  and  $\delta$  along with that of several other enzymes and factors. In this report, we describe studies performed with intact MCF7 cells and the DNA synthesome isolated from MCF7 cells. In our report we directly compared the effectiveness of dFdCTP and araCTP as inhibitors of the DNA synthetic process. Our results showed that dFdCTP is significantly more potent than araCTP in the *in vitro* SV40 DNA replication assay. Full-length DNA was produced in the presence of very low levels of both drugs, suggesting that incorporation of dFdCTP and araCTP did not stop the polymerases from elongating the DNA template. Our results were in agreement with those of Ross et al. (1995), who demonstrated that dFdC was progressively incorporated into nascent DNA of increasing size in intact HL60 cells [28]. In contrast, studies of dFdCTP incorporation using *in vitro* primer extension assays by purified DNA polymerase  $\alpha$  and  $\delta$  demonstrated that after incorporation of dFdCTP to the 3' terminus of the elongating DNA strand, one more deoxynucleotide can be added before the DNA polymerases are unable to continue elongating the nascent strand. Therefore dFdC appears to act as a chain terminator [15].

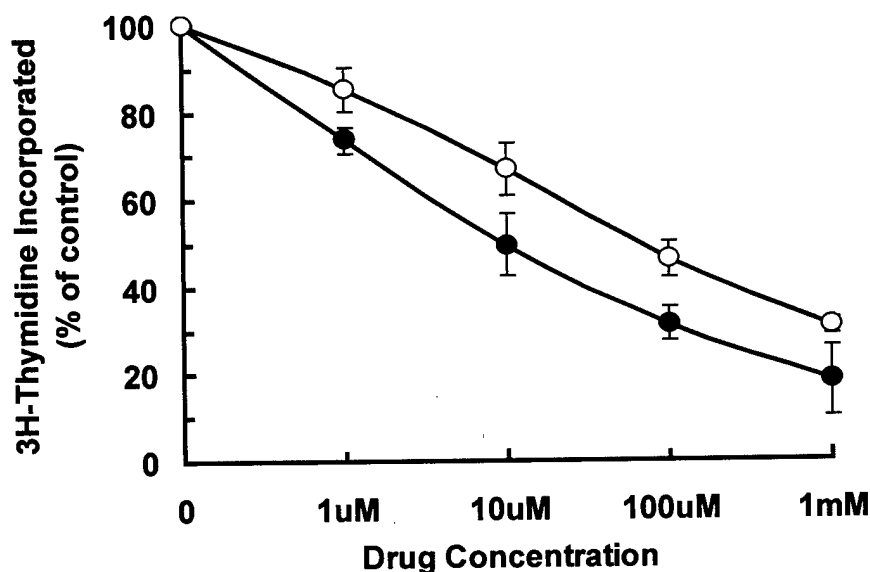
Gemcitabine is more potent than araC for several reasons. First, the chemical addition of fluorine atom to the 2' position of the furanose ring of the drug makes dFdC

more lipophilic and therefore more permeable to cells than araC. Second, deoxycytidine kinase has a higher affinity for dFdC than araC which leads to higher levels of dFdCTP than araCTP in cells. Third, dFdC inhibits ribonucleotide reductase which causes depletion of the cellular pools of deoxynucleoside triphosphate, particularly, the levels of the competing metabolite, dCTP. Thus, the ratio of cellular dCTP to dFdCTP favors the inhibition of DNA synthesis by dFdCTP (5,11). In contrast, araC has no effects on dNTP pools and is not known to act as an inhibitor of ribonucleotide reductase which helps maintain higher levels of active metabolite within the cell. Finally, the elimination of cellular dFdCTP is slower than araCTP [15]. All of these characteristics of dFdCTP contribute to the higher intracellular concentration of dFdCTP as compared to araCTP. dFdCTP is found at from 9- to 20-fold higher concentrations in treated cells than is in cells treated with equivalent concentrations of araC [10]. However, the increase in the intracellular concentration of dFdCTP does not completely account for the greater cytotoxicity of dFdCTP in intact cells, which has been reported to be 180-fold more toxic than araC (5).

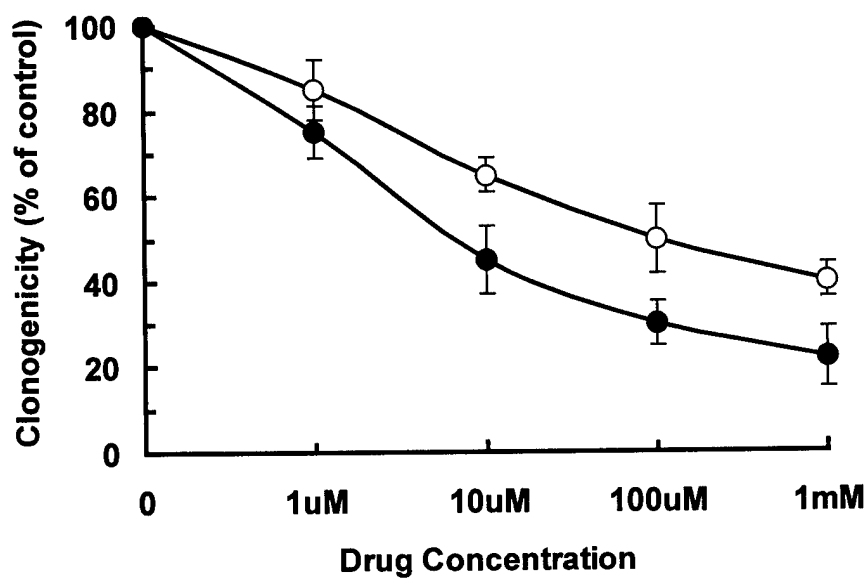
In conclusion, in this report we have demonstrated that dFdCTP is significantly more potent than araCTP at inhibiting the DNA synthetic process in our cell-free SV40 *in vitro* DNA replication assay system employing the M7DS. The results of this study verify that the DNA synthesome can serve as a relevant *in vitro* model system for studying the mechanism of action of anticancer drugs that directly affect DNA synthesis, and that the mechanisms through which these drugs inhibit *in vitro* DNA synthesis closely parallel the inhibitory effects of these drugs in intact cells [1,3b,9,31,32,] We have demonstrated in our laboratory that the DNA synthesome is able to incorporate araCTP into internucleotide linkages, and that this incorporation of araC into internucleotide linkages more closely resembles the molecular events occurring in intact cells than can be achieved using purified DNA polymerases [31,32].

As proposed in the statement of work, we are currently setting up two-dimensional high-resolution electrophoresis system to further examine the types of daughter DNA molecular produced by the M7DS. We also in the initiation stage to study the kinetics of replication and coordinated DNA synthesis mediated by DNA synthesome. Continued analysis of the mechanisms by which these drugs mediate cytotoxic effects will uncover the effects the drugs has on the initiation, elongation and termination stages of the DNA synthesis process. Thus, our *in vitro* model system, which utilizes the DNA synthesome to mediate the DNA synthetic reaction is anticipated to be of substantial value for gaining insight into the mechanism(s) of action of dFdCTP and other anticancer drugs that directly inhibit cellular DNA synthesis. The continued validation of the *in vitro* DNA replication model system employing the DNA synthesome is therefore expected to be of considerable value in the search for more effective anticancer drugs than conventional model systems that only employ highly purified individual enzymes such as DNA polymerases or topoisomerase II.

## FIGURES

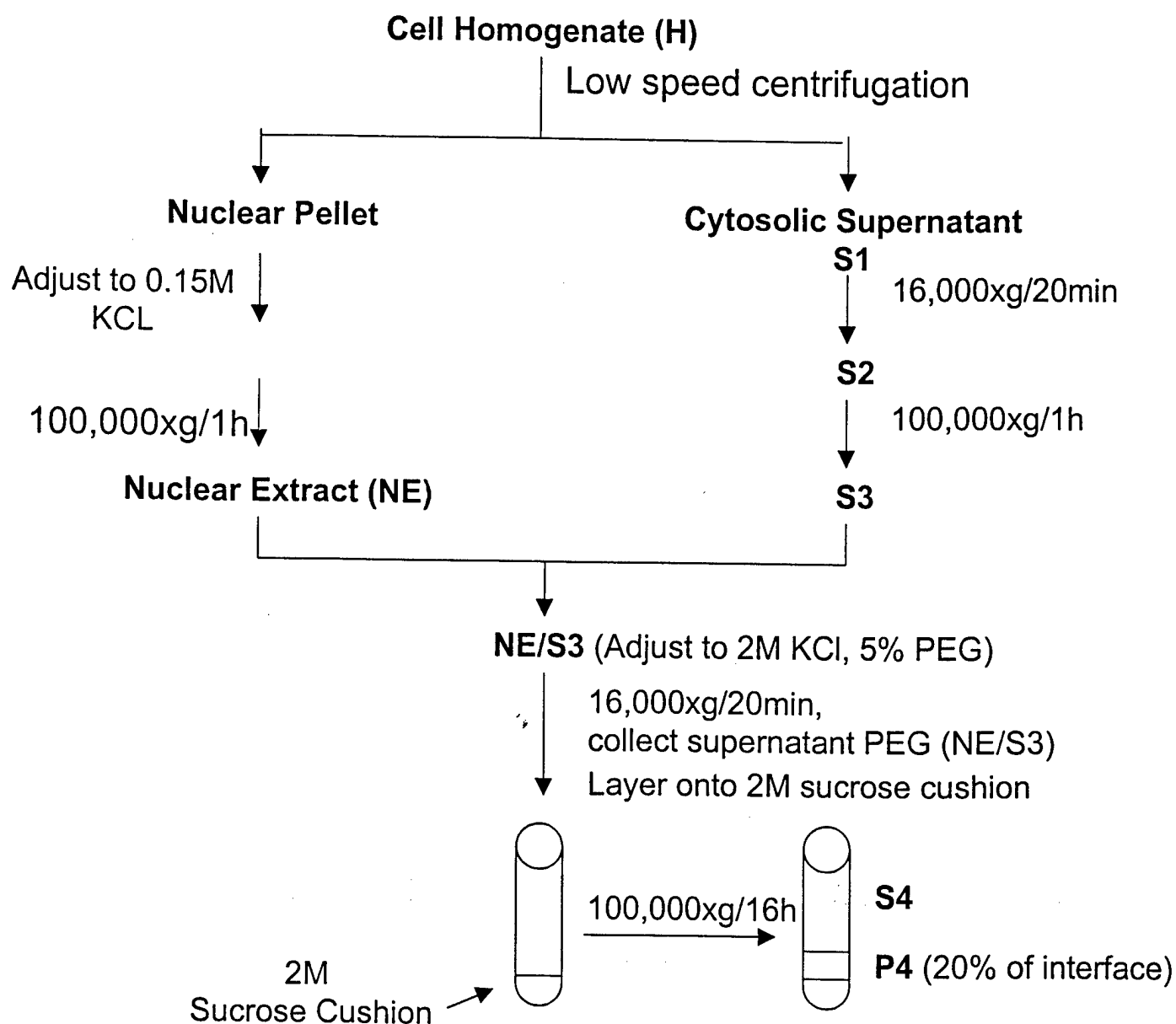


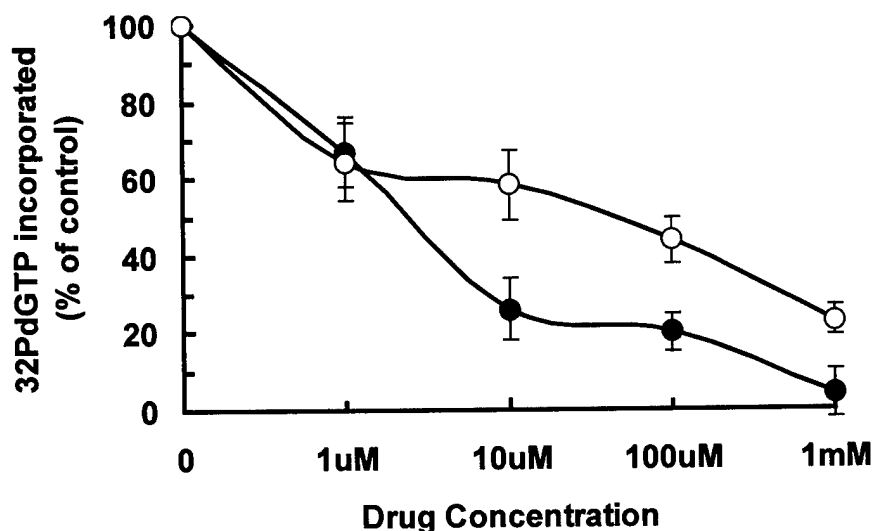
**Figure 1. Effect of dFdC and araC on intact MCF7 cells DNA synthesis:** MCF7 cells ( $5 \times 10^4$ ) were seeded onto 60mm cell culture plates and incubated for 24 hours at  $37^\circ\text{C}$  in Joklik's modified Eagle's medium. The cells were then exposed to one of several different concentrations of the indicated drug for 24 hours at  $37^\circ\text{C}$ . The cells were then labeled with  $^3\text{H}$ -thymidine ( $1\mu\text{Ci/ml}$  of medium). After a 4-hour incubation, the cells were lysed and the level of DNA synthesis was measured by quantifying the amount of  $^3\text{H}$ -thymidine present in acid insoluble material (○: araC, ●: dFdC). Each point represents the average of three separate experiments; error bars represent standard error of the mean. Cells grown and labeled in the absence of drug served as the controls to which the drug-treated cells were compared.



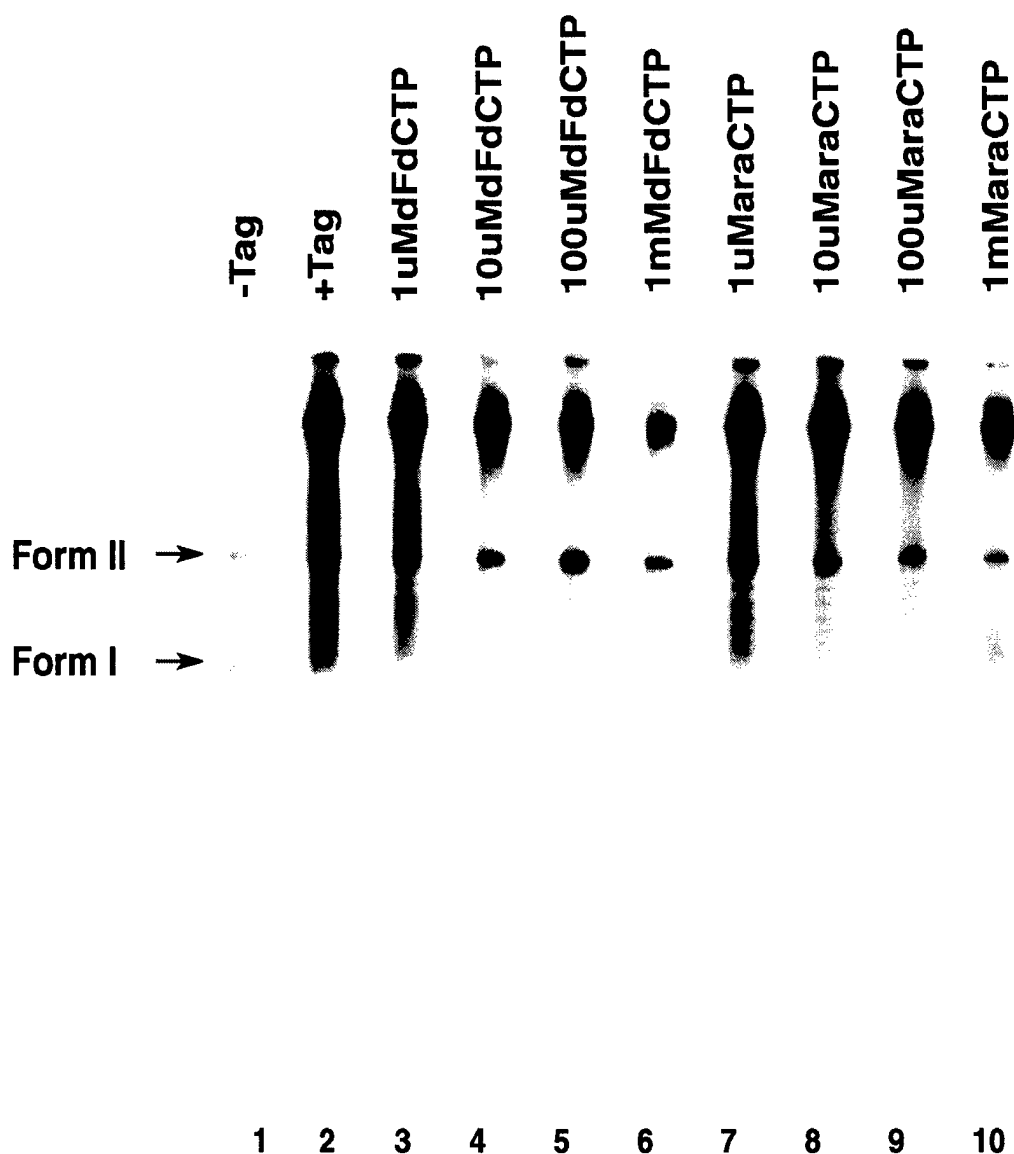
**Figure 2. Effect of dFdC and araC on the clonogenicity of intact MCF7 cells:** MCF7 cells ( $10^3$ ) were seeded onto 60mm cell culture plates and incubated for 24 hours at 37°C in Joklik's modified Eagle's medium. Cells were then exposed to different concentrations of drugs for 4 hours followed by incubation in drug-free medium for 5 days. Colonies were fixed with 10% formaldehyde in phosphate-buffered saline and visualized by Giemsa stain and counted (○: araC, ●: dFdC). Each point represents the average of three separate experiments; error bars represent standard error of the mean. Cells grown and labeled in the absence of drug served as the controls to which the drug-treated cells were compared.

**Figure 3. Flow diagram of the subcellular fractionation scheme used to partial purify the DNA synthesome**

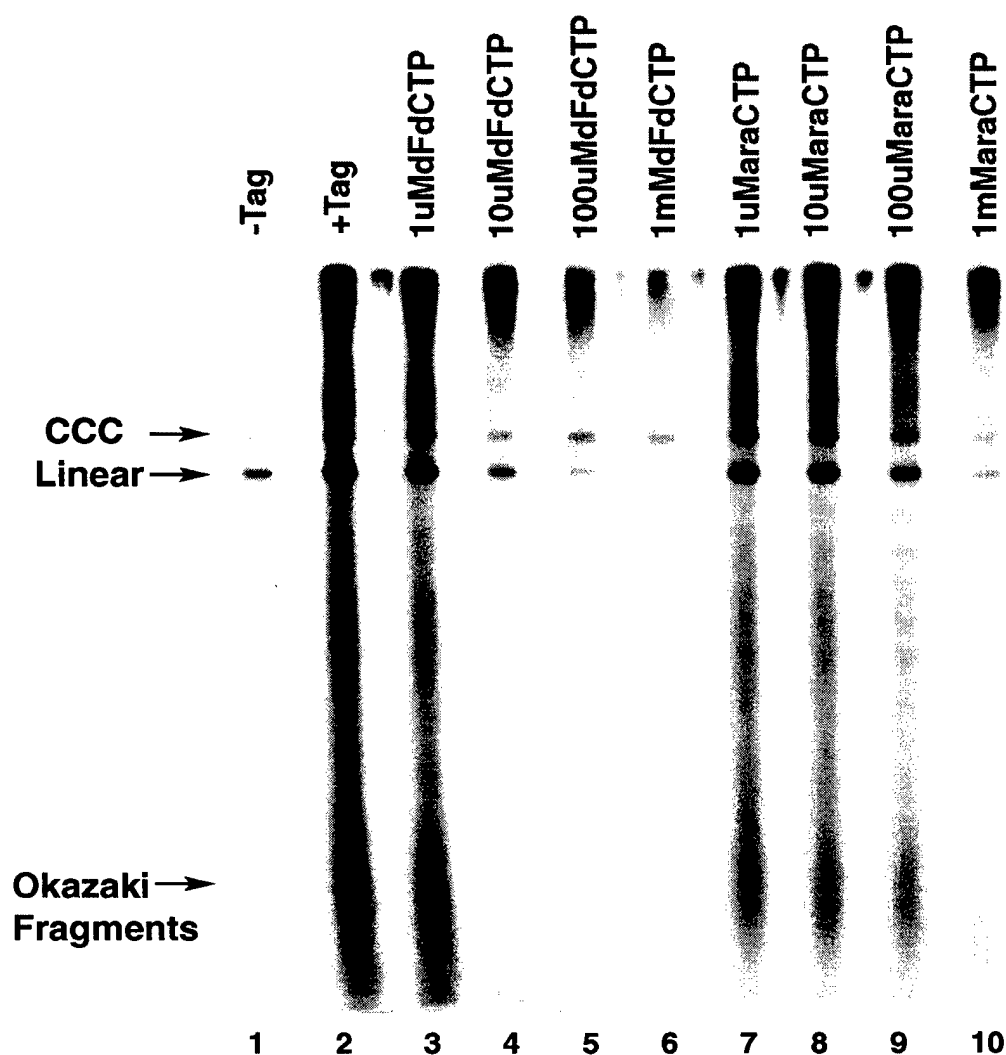




**Figure 4. Effect of dFdCTP and araCTP on DNA synthesesome mediated *in vitro* SV40 DNA replication.** The assay was performed essentially as described in Malkas et al.[9] in the absence or presence of increasing concentration of drug. The reaction mixture (25  $\mu$ l) contained 30mM HEPES (pH 7.5), 7mM  $MgCl_2$ , 0.5mM DTT, 2.5-3 $\mu$ g SV40 large T-antigen, 20 $\mu$ g of synthesesome protein fraction, 50ng of the plasmid pSVO<sup>+</sup> containing an inserted SV40 replication origin DNA sequence, 1 $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]dGTP (New England Nuclear, 3000 Ci/mmol), 100 $\mu$ M each of dATP, and dTTP, 10 $\mu$ M each of dCTP and dGTP, 200 $\mu$ M each of rCTP, rGTP, and rUTP, 4mMrATP, 40mM phosphocreatine, and 1 $\mu$ g creatine phosphokinase. The replication reaction was started by incubating the reaction mixture at 37°C for 4 hours. One microliter of the reaction mixture was spotted onto Whatman DE81 filters. The filters were then processed to quantify the amount of radiolabelled nucleotide incorporated into the DNA template [9]. Each point represents the average of three separate experiments; error bars represent standard error of the means (○: araC, ●: dFdC). Cells grown and labeled in the absence of drug served as the controls to which the drug-treated cells were compared.



**Figure 5: Neutral agarose gel analysis of the reaction products of the *in vitro* SV40 DNA replication assay.** The DNA replication products formed in the *in vitro* DNA replication reaction were isolated by phenol/chloroform extraction followed by precipitation at room temperature with 2-propanol in the presence of 2M ammonium acetate. The isolated DNA was resuspended in 10mM Tris/1mM EDTA, and the reaction products were resolved from one another using 1% agarose gels under neutral condition. The gels were dried and exposed to Kodak XAR5 films at  $-80^{\circ}\text{C}$  for 8 hours.



**Figure 6: Alkaline agarose gel analysis of the reaction products of the *in vitro* SV40 DNA replication reaction.** The DNA replication products formed in the *in vitro* DNA replication reaction were isolated by phenol/chloroform extraction followed by precipitation at room temperature with 2-propanol in the presence of 2M ammonium acetate. The isolated DNA was resuspended in 10mM Tris/1mM EDTA, and the reaction products were resolved from one another using 1% agarose gels under alkaline condition. The gels were dried and exposed to Kodak XAR5 films at  $-80^{\circ}\text{C}$  for 8 hours.

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## **APPENDIX**

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**Key research accomplishment:**

- **dFdC was more potent than araC at inhibiting intact MCF7 cell DNA synthesis and clonogenicity.** [<sup>3</sup>H]-Thymidine incorporation was inhibited by 50% at a dFdC concentration of 10uM, which was about 10-fold lower than the concentration of araC required to inhibit intact cell DNA synthesis by the same amount. As examined by clonogenicity assay, dFdC was also significantly more cytotoxic than araC after a 24-hour incubation.
- **dFdCTP was more potent than araCTP at inhibiting *in vitro* SV40 DNA synthesis mediated by DNA synthesome derived from MCF7 cells.** In the presence of 10uM dCTP, 8uM dFdCTP and 80uM araCTP were required to inhibit *in vitro* SV40 DNA synthesis by 50%.
- **dFdCTP and araCTP affected the formation of MCF7 DNA synthesome replication intermediates.** *In vitro* SV40 replication assays demonstrated that the formation of full-length DNA along with replication intermediates were inhibited by dFdCTP in a concentration-dependent manner. However, full-length DNA was produced in the *in vitro* DNA replication assay even when the dFdCTP was incubated in the assay at concentrations of up to 1 mM.
- **The MCF7 cell DNA synthesome can serve as a relevant model to study the mechanism of action of anticancer drugs that directly affect DNA synthesis.**

**#2002 Balance between apoptotic (AI) and proliferative (PI) indices in gemcitabine-treated HCT116 colon carcinoma mouse xenografts.** Wexler, E.J., Czerniak, P.M., Slee, A.M., and Kerr, J.S. *General Pharm. DuPont Pharmaceuticals, Wilmington, DE 19898.*

Tumor growth is dependent on the balance between apoptosis and proliferation. How therapeutic agents affect this balance is often unknown. Gemcitabine, a new anticancer drug with novel metabolic properties, has antitumor effects that vary with dosing schedule. These properties make it a useful tool to characterize the apoptotic/proliferative balance in tumors. When dosed Q1D $\times$ 5 at 3.5 mg/kg/day and Q3D $\times$ 5 at 120mg/kg/day, tumor growth in the HCT116 xenograft was reduced 73% and 92% ( $p < 0.05$ ), respectively. AI was determined using TUNEL immunohistochemistry *in situ*, and PI was measured by bromodeoxyuridine (BrdU) incorporation. Composite images of tumor sections were quantitated for areas of necrosis, proliferation and viability using an M2 MCID Image Analysis System (Imaging Resources). AI in controls was  $2.4\% \pm 0.13$  (Mean  $\pm$  SEM), and significantly ( $p < 0.05$ ) increased with both dosing regimens,  $5.7\% \pm 0.06$  with Q1D $\times$ 5, and  $3.5\% \pm 0.02$  in the Q3D $\times$ 5 group. PI in controls was  $28.1\% \pm 1.9$  with no change in PI with daily dosing ( $27.6\% \pm 1.9$ ) but Q3D $\times$ 5 dosing led to a 26% reduction in PI to  $20.8\% \pm 3.5$  ( $p < 0.05$ ). Although tumors from animals treated with gemcitabine were significantly smaller than controls, the percent of viable cells normalized for total tumor area was increased by 22–35%. These data demonstrate that AI and PI may be used to characterize the intratumor dynamics associated with different dosing regimens.

**#2003 Analogs of phenylbutyrate as mechanistic probes of differentiation.** Ludeman, S.M., Carducci, M.A., Springer, J.B., Kruszewski, M., Shulman-Roskes, E.M., and Colvin, O.M. *Duke Comprehensive Cancer Center, Durham, NC 27710 and The Johns Hopkins Oncology Center, Baltimore, MD 21205.*

Phenylbutyrate (PB) has emerged in phase 1 trials as a promising differentiation agent. Most literature reports refer to PB as a prodrug for phenylacetate (PA) with the conversion occurring through  $\beta$ -oxidation. To investigate this hypothesis, a series of PB analogs were synthesized which were incapable of undergoing a  $\beta$ -oxidation mechanism. In addition, one analog did not have an acid functionality but in its place had a group which was electronically similar to a carboxylate moiety. Each of these analogs (benzyloxyacetate, 2,2-difluoro-4-phenylbutyrate and 1,1,1-trifluoro-5-phenyl-2-pentanone) were screened for cell growth inhibition against five lines of human prostate cancer and each had activity that either paralleled or was better than that of PB. Relative to PB, significantly lower  $IC_{50}$  values were also achieved for the analogs against multiple cell lines. These preliminary results suggest that (1)  $\beta$ -oxidation is not intricate to PB activity and (2) PB may have a mechanism of action which is unique in comparison with that of PA and/or butyrate.

**#2004 Selection and characterization of a CHO cell line resistant to bis(ethyl) polyamine analogues.** McCloskey, D.E., and Pegg, A.E. *Dept. of Cellular and Molecular Physiology, Penn State University College of Medicine, Hershey, PA 17033.*

A CHO cell line that is resistant to bis(ethyl) polyamine analogues has been selected using  $N^1,N^{12}$ -bis(ethyl)spermine [BE3-4-3]. The parental CHO cell line used was the C55.7 CHO cell line which is auxotrophic for putrescine because of a point mutation which rendered ornithine decarboxylase inactive [Somat Cell Genet. 1985, 11(1):11–23]. Cells were exposed to BE3-4-3 for 120 h and then placed in drug-free medium and allowed to recover exponential growth. Four cycles of treatment with 10  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M, and 10  $\mu$ M BE3-4-3, respectively, were carried out. The resulting cells (C55.7Res) were resistant to at least 100  $\mu$ M BE3-4-3 with growth in the presence of the polyamine analogue similar to that of the parental cells cultured in drug-free medium. Characterization of the C55.7Res cells indicate that they are cross resistant to  $N^1,N^{11}$ -bis(ethyl)norspermine [BE3-3-3] with  $IC_{50}$  values of 7  $\mu$ M and 80  $\mu$ M, respectively for C55.7 and C55.7Res. The resistance to BE3-3-3 has remained stable through 20 passages in drug-free medium, indicating a heritable alteration in the C55.7Res cells. The C55.7Res cells are not cross-resistant to methylglyoxal bis(guanyldiazide), chlorobutrin or cisplatin. Intracellular accumulation of BE3-3-3 is similar to that of the parental cell line, indicating that the resistance is not a result of altered drug transport. Preliminary studies indicate that the polyamine pools of the C55.7Res cells are altered and that spermidine/spermine  $N^1$ -acetyl transferase activity is not induced by BE3-3-3 concentrations up to 25  $\mu$ M whereas there is  $>250$  fold induction in C55.7 cells at 25  $\mu$ M. Further characterization of this cell line may lead to better understanding of the mechanisms of action of the polyamine analogues. Support by GM-26290.

**#2005 A novel DNA minor groove binder MS-247: Design, synthesis and antitumor activity against human cancer xenografts.** Matsunaga A., Komatsu H., Kohno H., Edatsugi H., Matsuba Y., Nakanishi O., Sato S., Yamori T., Tsuruo T. *Life Sciences Laboratory, Mitsui Chemicals Inc., Chiba, Japan; Institute of Biological Science, Mitsui Pharmaceuticals, Inc., Chiba, Japan; Japanese Foundation for Cancer Research, Tokyo, Japan.*

MS-247 (2-[N-[1-methyl-2-[5-[N-[4-[N,N-bis(2-chloroethyl)amino]phenyl]carbamoyl]-1H-benzimidazol-2-yl]pyrrol-4-yl]carbamoyl]ethylidimethylsulfonium dip-toluenesulfonate) is a new synthetic anticancer agent with a novel DNA binding moiety, based on the structure of typical DNA minor groove binder Netropsin, and a DN A alkylating phenyl mustard residue. MS-247 showed significant cytotoxicity

in several murine tumor cell lines and showed strong *in vivo* antitumor activity against murine tumor model systems. MS-247 was also effective against 38 human cancer cell lines. We further examined the antitumor activity of MS-247 against a panel of 17 human cancer xenografts, including lung, colon, stomach, breast and ovarian cancers. Antitumor activities of MS-247 were significant and higher than those of adriamycin and cisplatin in all 17 xenografts. Among these cancers, a marked antitumor activity of MS-247 was observed in lung cancers (NCI-H23: T/C 4%, DMS114: T/C 19%, DMS273: T/C 2%). MS-247 was also effective against a paclitaxel-insensitive cancer, HCT15, and CPT-11-insensitive cancers, A549, HBC-4 and SK-OV-3. These results suggest that MS-247 can be a candidate for clinical investigation.

**#2006 The DNA synthesome: Extensive purification and serve as a model for studying the mechanism of anticancer drugs.** HaiYan Jiang, Robert J. Hickey, Philip W. Wills, Timothy, D. Tom and Linda H. Malkas. *Department of Pharmacology and Experimental Therapeutics, University of Maryland, Baltimore, School of Medicine.*

DNA replication enzymes and factors have been found involved in cell cycle control and cancer formation. The concept that many enzymes and factors involved in the mammalian DNA replication function together as an organized multiprotein complex has been supported by increasing evidence. We have purified a discrete multiprotein complex containing DNA polymerase  $\alpha$  from human cancer HeLa and MCF7 cell lines. An enrichment of this complex was seen through the purification steps and this DNA polymerase  $\alpha$  containing complex copurifies with peak DNA polymerase  $\alpha$  and *in vitro* simian virus 40(SV40) DNA replication activity. We have designated this complex the DNA synthesome. Finally, we purified the DNA synthesome by electroeluting this complex from native polyacrylamide gel. Further analysis of the DNA synthesome showed it contains DNA polymerase  $\delta$ , proliferating cell nuclear antigen (PCNA), replication protein A (RPA), topoisomerase I and topoisomerase II, which are essential components for DNA replication. Taken together our evidence indicates that the DNA synthesome represents the fundamental DNA replication unit of the human cell. Finally, The DNA synthesome was used as *in vitro* model system to investigate the mechanisms of action of cytarabine(ara-C) and gemcitabine(dFdC), which directly affect cellular DNA replication. This work was supported in part by grant #CA57350 and CA73060 to LHM and CA74904 to RJH. JHY is a recipient of DOD Breast Cancer Research Fellowship.

## PREVENTION/BASIC SCIENCE AND CLINICAL STUDIES 3: Breast and Prostate Cancer Chemoprevention

**#2007 Altered urinary excretion of 2-OH-estrone to 16-OH-estrone in women after soya isoflavone consumption.** Cree, M., Josyula S., Anderson, K.E., Nagamani, M. and Lu, L.-J.W., *The University of Texas Medical Branch, Galveston, TX 77555.*

Women consuming a traditional Asian diet high in soy have a lower rate of breast cancer than women consuming a western diet low in soy content. We investigated the effect of soya isoflavones on a putative breast cancer risk marker, i.e., urinary ratios of anticarcinogenic 2-OH-estrone (2OHE1) to carcinogenic 16-OH-estrone (16OHE1). Eight premenopausal women in a metabolic unit were placed on an isocaloric, soya diet containing 150 mg isoflavones daily for one complete menstrual cycle and 4 months later on another soya diet without isoflavones. Overnight 12 hour urine was analyzed for 2OHE1 and 16OHE1 using a commercial ELISA kit. Results showed that a diet rich in isoflavones increases the levels of 2OHE1 ( $16.96 \pm 2.96$  nmol/12 h with isoflavones,  $11.61 \pm 2.03$  without isoflavones,  $p < 0.01$ ), had no effect on the formation of 16OHE1 ( $7.72 \pm 1.25$  nmol/12 h with isoflavones,  $6.99 \pm 1.14$  without isoflavones,  $p < 0.2$ ). Urinary ratios of 2OHE1 to 16OHE1 were higher while subjects were on the isoflavone-containing soya diet ( $2.57 \pm 0.35$ ) than on the isoflavone-free soya diet ( $1.99 \pm 0.32$ ,  $p < 0.005$ ). In summary, soya isoflavones may prevent breast cancers by increasing the levels of anti-carcinogenic metabolites of 17 $\beta$ -estradiol. Supported by USPHS CA65628, CA56273, CA45181, NIH NCRR GCRC MO1 RR00073, and AICR 95B119.

**#2008 Reduction of estrone sulfate levels in premenopausal women after soya consumption.** Lu, L.-J.W., Anderson, K.E., Grady, J.J., and Nagamani, M. *The Univ. of Tex. Med. Branch, Galveston, TX 77555.*

Soybean (soya) consumption is associated with reduced rates of breast cancer; this oncoprotective effect may be due in part to reduced circulating ovarian steroids (CEBP 5: 63-70, 1996). We further determined that these reductions were observed for the entire cycle and are not mediated through the interaction of phytoestrogen isoflavones with gonadotropins. We examined the effect of soya diet on the levels of estrone sulfate which is the most abundant circulating estrogen and is a good index for estrogenicity. Eleven premenopausal women ingested 36-oz portions of soymilk containing ~150 mg of daidzein and genistein daily for one menstrual cycle. Their daily serum estrone sulfate levels were analyzed by specific radio-immunoassay. Approximately 30% and 40% de-

Resistance to methotrexate (MTX), an important cause of treatment failure for children with acute lymphoblastic leukaemia (ALL), can result from reduced intracellular drug accumulation. Cloning of the Reduced Folate Carrier (RFC) gene, encoding a putative MTX-transport protein, has allowed characterization of molecular changes leading to defective MTX transport. The structure of the RFC gene in transport-defective MTX-resistant pediatric leukaemia cells, CEM/MTX R, was compared to parental CCRF-CEM cells. Sequencing of the entire coding region identified a single point mutation (G → A) in CEM/MTX R cells resulting in a Glu → Lys substitution. Western analysis of CEM/MTX R membrane preparations indicated greatly reduced levels of RFC by comparison with wild-type cells. The full length RFC cDNA was electroporated into CEM/MTX R cells, and transfectant cells over-expressing the wild-type RFC gene were characterized. By comparison with the vector-only control, there was a 125-fold reversal of MTX resistance in the transfectant cells, while response to the lipophilic antifolate, trimetrexate, was unaffected. Surprisingly, there was no reversal of resistance to tomudex, another antifolate transported by RFC, in the transfectant cells. The results indicate that alterations in the RFC gene may contribute to transport-related MTX resistance in childhood ALL, but that additional mechanisms can mediate resistance to antifolate compounds.

**#4459 Characterization of RNA binding by mutant variants of human dihydrofolate reductase.** McPherson, J.P., Sauerbrey, A., Meehl, M., Russo, A., Skacel, N., and Bertino, J.R. *Program of Molecular Pharmacology and Therapeutics, Memorial Sloan Kettering Cancer Center, New York, NY 10021.*

Previous studies have shown *in vitro* that human dihydrofolate reductase (hDHFR) regulates its own synthesis through negative feedback at the level of protein translation. The autoregulatory nature of this mechanism has been postulated to be due to the specific binding of the enzyme to its own mRNA. Both translational inhibition and RNA binding by hDHFR are attenuated in the presence of the antifolate inhibitor methotrexate (MTX). In order to understand the role of antifolates in RNA binding and translational inhibition, we have compared the RNA binding of native hDHFR to that of mutants with impaired binding to MTX. Native hDHFR and mutant variants with substitutions at leu22 and/or phe31 were purified as hexahistidine fusion proteins by Nickel affinity chromatography and RNA binding was examined using electrophoretic mobility-shift assays. Native hDHFR protein (nM) bound avidly to its mRNA (pM) and this binding was decreased in the presence of nM concentrations of MTX and was nearly abolished at  $\mu$ M concentrations. In contrast,  $\mu$ M concentrations of 5-fluorouracil only slightly impaired RNA binding. Mutant variants with substitutions at leu22 and/or phe31 were observed to retain RNA binding in the absence of drug. However, MTX-dependent attenuation of RNA binding by these mutant enzymes was found to require 10- to 100-fold higher concentrations of MTX compared to native hDHFR. Ongoing studies will determine whether these mutants also demonstrate differences in their ability to regulate DHFR synthesis.

**#4460 Ara-C affects the formation of cancer cell DNA synthesome replication intermediates.** Abdel-Aziz, W.; Jiang, H.Y.; Hickey, R.J.; and Malkas, L.H. *Department of Pharmacology and Experimental Therapeutics, UMB, Baltimore, MD 21201.*

Our laboratory was the first to isolate and characterize a multiprotein DNA replication complex (DNA synthesome) from human as well as from murine mammary carcinoma cells. DNA synthesome supports the *in vitro* replication of DNA containing the papovavirus origin of replication in the presence of the viral large T-antigen. We are currently using the *in vitro* replication assay to study the mechanism of action of anticancer drugs affecting DNA replication. In order to directly validate that the *in vitro* interaction of ara-C with the DNA synthesome is representative of the molecular events occurring in intact cells, the types of daughter DNA molecules produced by the synthesome in the presence and absence of ara-CTP were analyzed and compared with papovavirus replication intermediates isolated from virus-infected cells exposed to ara-C. Our results using different concentrations of ara-CTP (1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M, and 1 mM) demonstrated that full length daughter DNA molecules were obtained in the presence of 1 and 10  $\mu$ M ara-CTP, while at higher concentrations (100  $\mu$ M and 1 mM), there was an inhibition of full length daughter DNA synthesis. Our data suggest that the initiation phase of DNA synthesis is inhibited by ara-CTP since the production of the short Okazaki fragments was inhibited at all concentrations of the drug. We have also found that increasing ara-C concentration was accompanied by a gradual decrease in  $^3$ H-thymidine incorporation in intact cells. The  $IC_{50}$  of ara-C for inhibition of *in vitro* DNA replication was comparable to that required for inhibition of intact cell DNA synthesis. Ara-CTP also inhibited the synthesome-associated DNA polymerases  $\alpha$  and  $\delta$ .

**#4461 Defining the mechanism of action of Ara-C using the human cell DNA synthesome as an *in vitro* model system.** Han, S.; Hickey, R.J.; Jiang, H.Y.; Malkas, L.H. *Department of Pharmacology and Experimental Therapeutics, UMB, Baltimore, MD 21201.*

The antimetabolite 1- $\beta$ -D-arabinofuranosylcytosine (ara-C) has been used as a highly effective agent for the treatment of leukemia. It has been shown to be a potent inhibitor of intact cell DNA synthesis, and its active metabolite (ara-CTP) is also a strong inhibitor of the activity of isolated DNA polymerases  $\alpha$ ,  $\delta$  and  $\epsilon$  *in vitro*. We have recently observed that the DNA polymerases  $\alpha$ ,  $\delta$ , and  $\epsilon$  are components of the DNA synthesome and the activities of these DNA polymerases

are differentially inhibited by ara-CTP. In this study, we further investigated how this anticancer drug disrupts the growth of daughter DNA molecules by interacting with the synthesome associated DNA polymerases. By using oligonucleotide-primed templates, we examined the ability of the synthesome associated DNA polymerases  $\alpha$  and  $\delta$  to insert ara-CTP into DNA and to bind to, (and to extend from) a DNA strand containing an ara-CMP 3' terminus. We found that the synthesome associated DNA polymerase  $\alpha$  could incorporate ara-CTP into the internucleotide positions but serves as a poor substrate for the addition of the next deoxynucleotide.

**#4462 Comparison of arabinosylcytosine and dideoxycytidine induced drug resistance in H9 cells.** Fernandez, M., Han, T., Yo, P., Wang, W., and Agarwal, R.P. *University of Miami, School of Medicine, Miami, FL 33101.*

Our laboratory has been involved with the studies of host cell factors causing resistance to antiviral and anticancer agents. This report compares resistance induced by the dideoxycytidine analogs, dideoxycytidine (ddC, an antiviral agent), and arabinosylcytosine (araC, an anticancer agent). H9-ddC0.5w, H9-ddC5.0w, H9-araC0.05w, and H9-araC0.5w cells were selected by propagating human lymphocytic H9 cells in the presence of 0.5  $\mu$ M ddC, 5.0  $\mu$ M ddC, 0.05  $\mu$ M araC and 0.5  $\mu$ M araC, respectively. In the resistant cells, the deoxycytidine kinase (dCK) activity was 48.5%, 35.9%, 54% and 31.1%, ddCTP concentrations 20.4%, 2.1%, 11.5%, and 0.4%; and araCTP concentrations 28.2%, 16.4%, 2.7% and 0.4% of control. The ddC resistant cells were 5.4-fold and 15.7-fold resistant to ddC. Whereas araC resistant cells were significantly resistant to araC (27.8-fold and >294-fold) the ddC resistant cells were negligibly resistant to araC (1.2-fold and 1.1-fold). Differences were noted in the phosphorylation of ddCDP and araCDP to their triphosphates. araCDP was phosphorylated to araCTP more efficiently than ddCDP to ddCTP. These observations suggest that, even though ddC and araC are analogs, there are differences in metabolism and toxicity in ddC resistant and araC resistant cells and conclusions drawn from one cell line may not be applicable to the other. (Supported by NIAID Grant # AI 29155)

**#4463 Ara-C resistance in leukemic lines confers cross-resistance or collateral sensitivity to other classes of anti-leukemic drugs.** Martin-Aragon, S., Fu, C., Solorzano, M., Ardi, V., and Avramis, V.I. *Division of Hematology/Oncology, School of Medicine, USC, Childrens Hospital LA, Los Angeles, CA 90027.*

The major limitation of antimetabolite drugs is that they produce drug-resistant clones *in vitro* and in patients who either do not respond or relapse soon after response has been documented. Leukemia patients in relapse are much more difficult to treat with other classes of antileukemic drugs. In order to understand this phenomenon of cross resistance, we used CEM/O and 5 CEM/ara-C-resistant leukemic clones to treat with drugs or radiation. 6-TG or 6-TGuo are cross resistant with ara-C from 1.1- to 15.8-fold using either MTT or a biology assay. Vincristine (VCR) showed cross-resistance with ara-C from 200- to 1E4-fold and only one resistant cell line, CEM/ara-C/G, was 3-fold more sensitive than CEM/O. This clone expresses p53 and does not overexpress bcl-2 protein. Taxotere, another mitotic inhibitor, showed cross-resistance, which was not dose dependent. Dexamethasone (DXM) also showed a significant degree of cross-resistance from 274- to 1E7-fold. The CEM/ara-C/G showed a 4.85E5-fold cross resistance of DXM with ara-C. Gamma radiation treatments up to 12.3 Gy showed a dose-dependent cross-resistance with ara-C from 1.43- to 2.42E5-fold. Only idarubicin was collaterally sensitive with ara-C from 4.6- to 218-fold in these cell lines. Other classes of anti-neoplastic drugs are being tested to further evaluate this phenomenon. It is apparent that ara-C resistance confers cross-resistance to many other classes of drugs and radiation, probably due to lack of apoptosis. This phenomenon may be significant in its adverse effects on the treatment of patients with relapsed leukemias. Efforts in determining the molecular mechanisms of cross-resistance in these cell lines are under investigation.

**#4464 Correlated overexpression of TS and FR- $\alpha$  in 5-FU resistant H630 cell lines.** Grmeiner, W.H., Kolar, C., Liu, J., and Lawson, T. *University of Nebraska Medical Center, Omaha, NE 68198-6805.*

Thymidylate synthase (TS) inhibition is central to cancer chemotherapy. TS is catalytically active as part of a ternary complex that includes the substrate dUMP and reduced folate. Thus, for TS overexpression to successfully overcome inactivation of normal levels of TS, the malignant cell must also have available increased levels of reduced folate. RT-PCR was used to amplify folate receptor (FR- $\alpha$ ) mRNA from H630, H630-1, and H630-10 cell lines. These are human colorectal cell lines and the -1 and -10 refer to the levels of 5-FU (in micromolar) to which they have been adapted in culture medium. JAR choriocarcinoma cells were included in this study because they had been previously shown to express FR- $\alpha$ . The results indicate that TS overexpression and FR- $\alpha$  overexpression positively correlate in the 5-FU resistant cell lines with levels ~5-fold higher in H630-1 and ~30-fold higher in H630-10 cell lines relative to H630 cells. Novel multimeric FdUMP (FdUMP[10]) agents have been developed in our laboratory and these have been conjugated with folic acid and show increased cytotoxicity to H630, H630-1 and H630-10 cell lines, relative to unconjugated multimers.

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### Complementation Between Polyomavirus Large T Mutants Affected in Viral DNA Replication and Transactivation of E2F Promoters

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There is a N-terminal domain common to all 3 polyomavirus T antigens that appears to be required for various aspects of cellular transformation. The biochemical activity associated with this domain is unclear but it may function as a DNA J domain interacting with members of the 70-kD heat shock protein family. We have generated a series of mutations affecting the putative J domain and analyzed the biological activities of the 3 T antigens. The mutations do not affect the properties of the middle and small T antigens but impair most of the properties of the large T antigen. This includes immortalization, transactivation of E2F-responsive promoters and viral DNA replication. Although the mutants are inactive in transactivation, they can bind to pRb. Interestingly, the transactivation defect can be complemented by dl-141, another large T mutant unable to transactivate and defective in pRb binding. We propose a model to explain the requirement for a DNA J domain in large T mediated viral DNA replication.

## 20

### Purification and Detailed Characterization of DNA Synthesome Using Combined Low Pressure Chromatography and Electroelution

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Strong evidence has been accumulated that most the enzymes and factors involved in the replication of mammalian DNA function together as an organized multiprotein complex. We have previously reported that a highly purified multiprotein form of DNA polymerase can be isolated from a variety of mammalian cell types and tissues. Native polyacrylamide gel electrophoresis of this purified multiprotein complex from HeLa cells revealed the presence of several high molecular weight multiprotein species. One of these complexes was readily recognized in Western blot analysis by a monoclonal antibody against the DNA replication essential protein DNA polymerase  $\alpha$ . In this report, we describe the most extensive purification of the HeLa cell DNA synthesome using combined low pressure chromatography and electroelution from native polyacrylamide gel. This most purified DNA synthesome showed DNA polymerase  $\alpha$ ,  $\delta$ , and high specific *in vitro* simian virus 40 (SV40) origin dependent DNA replication activity. Furthermore, Western blot showed that this DNA synthesome contains all of the enzymes required for DNA replication.

## 21

### Defining the Mechanism of Action of Anticancer Drugs Using the Human Cell DNA Synthesome as an *In Vitro* Model

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The multi-protein DNA replication complex (DNA synthesome) from human breast cancer cells can serve as a unique and novel *in vitro* system to investigate the mechanism of action of anticancer drugs. The antimetabolite 1- $\beta$ -arabinofuranosylcytosine (ara-CTP) has been used as a highly effective agent for the treatment of leukemia. It has been shown to be a potent inhibitor of intact cell DNA synthesis and the *in vitro* DNA polymerase activity of the isolated DNA polymerases  $\alpha$ ,  $\delta$  and  $\epsilon$ . Using the highly purified DNA synthetic apparatus (i.e. the DNA synthesome), we have shown that DNA polymerase  $\alpha$ ,  $\delta$ , and  $\epsilon$  are components of the synthesome and that their activity is differentially inhibited by ara-CTP. Furthermore, the inhibition of the DNA synthesome associated DNA polymerase  $\alpha$  activity increase in a concentration dependent manner, and correlated closely with the inhibition of SV40 origin dependent *in vitro* DNA replication. In contrast to the effect on polymerase  $\alpha$ , DNA synthesome associated polymerase  $\delta$  and  $\epsilon$  were not significantly inhibited by ara-CTP. We also have shown that the synthesome associated DNA polymerase  $\alpha$  could incorporate ara-CTP into the internucleotide positions, but ara-CTP serves as a poor substrate for the addition of the next deoxynucleotide. Our results suggest that inhibition of the activity of the mammalian cell DNA synthesome by ara-CTP is primarily due to primarily the inhibition of synthesome associated polymerase  $\alpha$ . The work was supported in part by grants from the National Institute of Health/NCI: # CA 57350 and CA73060 to L.H.M., and CA74904 to R.J.H.